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ARTICLE

Chitosan elicited immune response reduces photosynthetic electron transport and ion channel activity in the guard cells of *Vicia*

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ABSTRACT It has been shown that a fungal elicitor chitosan (CHT) inhibits the blue light-induced stomatal opening and can trigger stomatal closure. These movements are related to the H^+-ATPase activity in the guard cell (GC) plasma membrane that affects the transport of osmotically active solutes. The ATP for proton pumping is supplied mostly from mitochondrial respiration; however, a partial inhibition by DCMU implies a role of GC photosynthetic electron transport in the ATP supply. In order to investigate whether CHT affects the photosynthetic ATP production of *Vicia* GCs, the light-dependence of the photosynthetic electron transport rate (ETR) of individual GCs was assayed. In addition, to test the possible effect of CHT on the activity of ion channels, GC protoplasts were investigated by patch clamp technique.
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KEY WORDS

chitosan
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GCs respond to the presence of microbes by narrowing stomatal pores following perception of microbe-associated molecular patterns (MAMPs) such as CHT, a deacylated derivative of a major fungal cell wall component chitin. CHT was found to inhibit the light-induced stomatal opening in tomato (Lee et al. 1999). This inhibition was suppressed by catalase and ascorbic acid suggesting reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), as signalling components of elicitor-inhibited stomatal opening.

CHT not only inhibits the light-induced stomatal opening but can also induce stomatal closure (Srivastava et al. 2009). CHT can probably activate the abscisic acid coupled signalling pathway, raising the level of ROS, especially H_2O_2, as well as nitric oxide (NO) and cytosolic free Ca^{2+}. The source of H_2O_2 was suggested to be the plasma membrane NAD(P)H oxidase, as diphenyleneiodonium chloride (DPI), a NAD(P)H oxidase inhibitor was found to prevent the CHT induced stomatal closure (Srivastava et al. 2009).

Stomatal opening and closure is related to the plasma membrane H^+-ATPase activity in GCs, as it energizes other coupled transport mechanisms. Experiments using potassium cyanide (KCN) have revealed that ATP, required for blue light-induced opening is supplied mostly from mitochondrial respiration (Parvathi and Raghavendra 1995); however, partial inhibition by DCMU implies a role of GC photosynthetic electron transport in the ATP supply in *Vicia faba* (Mawson 1993). On the other hand, photosynthetic activity of GCs is essential in the production of NADPH and ATP utilized for $malate^{2-}$ synthesis in the cytosol (Shimazaki et al. 2007), where $malate^{2-}$ functions as an osmoticum in guard cells.

In the present work we examined whether CHT affects stomatal opening and closure in the model plant *Vicia faba*. We also studied whether this action involves a reduction in the photosynthetic activity of GCs. Fluorescent probes were used to determine the production and the cellular localisation of signalling components H_2O_2 and NO upon the CHT treatment.

Materials and Methods

Plant material and experimental solutions

Abaxial epidermal strips of completely unfolded leaves from 2 to 3 week-old broad bean (*Vicia faba* L. cv. Mirna) plants were used in all experiments. The plants were grown hydroponically in a controlled environmental chamber (Fitoclima S 600 PLH, Aralab, Portugal) under 12h light/ 12-h dark and 25/20°C cycle.

CHT (Sigma-Aldrich, Budapest, Hungary) was dissolved in 100 mM acetate (ACT) buffer pH 3.63 (Shepherd et al. 1997), to a concentration of 10 mg ml^{-1}. The stock solution was then used to prepare the experimental solution containing 100 µg ml^{-1} CHT, 1 mM ACT, 10 mM MES, 10 mM KCl and 100 µM $CaCl_2$ (pH 6.15 with KOH). The experimental solution without CHT was used as a control in all measurements.

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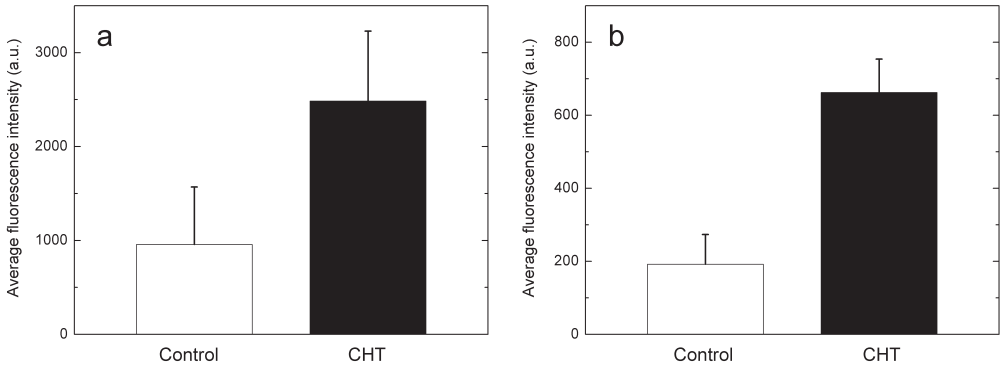

Figure 1. Average fluorescence intensity of the specific H_2O_2 probe AR (a) and the NO indicator DAF-FM (b) in *Vicia faba* GC chloroplasts of control and CHT treated leaves.

Stomatal aperture measurements

The width of stomatal apertures was measured on digital images taken from freshly peeled epidermal strips with the Image-Pro Plus 5.1 software. Experiments were repeated on three different days.

Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence of 2-3 stomata from abaxial epidermal peels was monitored with microscopy-pulse amplitude modulation chlorophyll fluorometer (Microscopy-PAM, Heinz Walz GmbH, Germany) mounted on an inverted epifluorescence microscope (Zeiss Axiovert 40, Zeiss GmbH, Germany). Instant light-response curves were obtained using the light-curve programme of the Microscopy-PAM, where actinic light intensity was increased in 8 steps during the 4 minutes of an experiment.

Localization of the fluorescent probes using confocal microscopy

Localization of the fluorescent probes in the abaxial epidermis of intact leaves was visualized using confocal laser scanning microscope (Olympus FV1000 LSM, Olympus Life Science Europa GmbH, Hamburg, Germany). H_2O_2 was detected by a specific probe 10-acetyl-3,7-dihydroxyphenoxazine (AR) (Amplex Red, Molecular Probes Invitrogen, Carlsbad, CA). NO was localised by the frequently used cell-permeable 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM, Sigma-Aldrich, Budapest, Hungary).

Electrophysiological recordings

GC protoplasts and experimental solutions were prepared as described previously (Horváth et al. 2002). Membrane currents in combination with the electrical parameters of the GC protoplasts were recorded in the conventional whole-cell configuration with software driven patch clamp amplifier (EPC 10, HEKA, Lambrecht, Germany).

Results and Discussion

Stomatal opening and closure use diverse set of signalling pathways and transporters (Kim et al. 2010). As CHT may act on different levels of signalisation in these processes, leaves were treated with CHT before stomatal opening at dawn and also by day.

CHT inhibits stomatal opening at dawn, arrests further opening and induce slight closure by day

Abaxial epidermis of broad been leaves were sprayed with the control and CHT containing solution in the dark at 6.00 am. After the treatment illumination was switched on and leaves of distinct plants were peeled hourly and digital images were taken immediately to determine the stomatal aperture sizes. At 6.00 am stomata were entirely closed with an average size of 5.1 ± 0.1 μm. During the illumination stomatal apertures of control plants increased and maximal opening was reached at 2.00 pm with an average size of 14.5 ± 0.5 μm. When the leaves were treated with CHT before 6.00 am stomata remained closed during the whole experimental period (6.4 ± 0.8 μm at 2.00 pm).

If the CHT treatment was applied at 10.00 am when stomata were partly opened (10.4 ± 1.2 μm), the apertures were not further increased but slightly reduced to 8.1 ± 0.5 μm until 2.00 pm. This is contrary to earlier results of Srivastava et al. (2009), who found at least 50% reduction in aperture sizes of pea stomata even by the application of a diluted 5 μg ml^{-1} CHT.

CHT induces both H_2O_2 and NO accumulation in GC chloroplasts and cytoplasm

H_2O_2 was found to be a component of GC signal transduction induced by CHT in tomato (Lee et al. 1999). In these experiments H_2O_2 was localised by the general ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). In

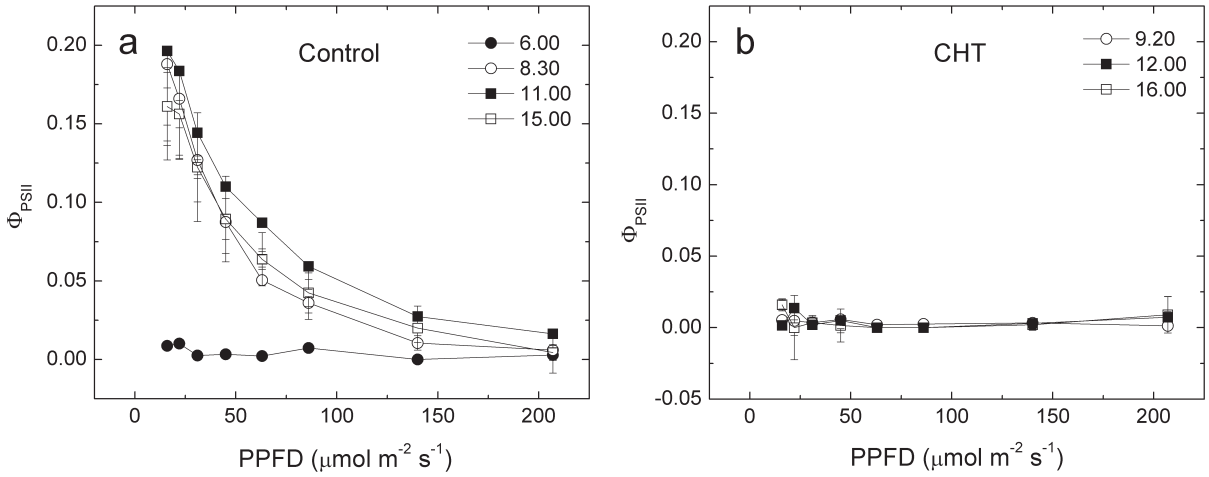

Figure 2. Φ_{PSII}-PPFD curves of stomata in epidermal strips of control (a) and CHT treated leaves (b) obtained during a day. Treatments were applied 30 min before the start of illumination at 6.00 am.

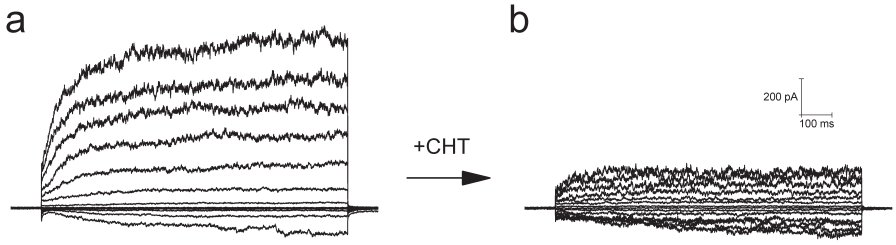

Figure 3. Whole-cell current responses to test voltages of a GC protoplast before and after the change of extracellular media to the 100 μg ml^{-1} CHT containing solution.

order to examine the production and subcellular localization of H_2O_2 upon CHT treatment in *Vicia faba* GCs, a specific fluorescent sensor AR was applied (Snyrychova et al. 2009). AR produces the highly fluorescent resorufin with H_2O_2, whose emission was detected between 585 and 610 nm using 543 nm HeNe laser excitation. Control and 100 μg ml^{-1} CHT treated leaves were infiltrated with 1 mg ml^{-1} AR through a pinhole made with a sharp pin. Leaves were kept in dark for 5 minutes and the infiltered epidermal regions were cut out and examined without peeling. We found that in CHT treated leaves H_2O_2 accumulates mainly in the chloroplasts and around the nucleus, which was quite surprising as NAD(P)H oxidase was suggested to be the source of ROS in the CHT induced stomatal closure (Srivastava et al. 2009). The average fluorescence intensity of AR in chloroplasts was significantly higher in CHT treated leaves compared the control ones (Fig. 1a).

The level of NO was monitored by the cell permeable fluorophore DAF-FM, which reacts with the nitrous anhydride N_2O_3, resulting by the oxidation of NO (Kojima et al. 1998) and produces a fluorescent N-nitrosilated form of diaminofluorescein. We found that CHT induced the production of NO, which accumulated in the chloroplasts (Fig. 1b) and the cytoplasm.

CHT hampers Φ_{PSII} thus photosynthetic ATP production available for stomatal opening

ROS and NO are essential signalling components in stomatal function. As we found their increased accumulation in GC chloroplasts after CHT treatment, their possible effect on photosynthetic electron transport and the coupled ATP production could not be excluded, as we found earlier in the case of NO in intact leaves (Wodala et al. 2008).

Therefore, the light-dependence of the operating quantum efficiency of PSII photochemistry (Φ_{PSII}) was assayed in order to ascertain whether CHT reduces the linear electron transport rate (ETR) thus GC photosynthetic ATP supply. Hence, after treating the leaves with the experimental solutions, epidermal strips were freshly peeled at given times and dark adapted in control solution for 15 min.

We found that GCs in strips peeled before the light period at 6.00 am exhibit extremely low Φ_{PSII} values at any actinic

light intensity (Fig. 2a). However, in parallel with stomatal opening in the morning, Φ_{PSII} increase and reaches its maximum around 11.00 am. On the contrary, Φ_{PSII} values in GCs treated with CHT at dawn before 6.00 am remained close to zero even at 16.00 pm (Fig. 2b).

Interestingly, when the leaves were treated with CHT during the daytime, when stomata were already open, Φ_{PSII} was not changed significantly (data not shown). From these facts it emerges that CHT reduces Φ_{PSII} and ETR thus photosynthetic ATP production available for stomatal opening when it is applied at dawn, but does not cause significant changes during the daytime. The latter accounts for the CHT-caused prevention of further opening and for the slight closure in *Vicia*.

Whole-cell currents of Vicia faba guard cell protoplasts are diminished by CHT

Vicia faba guard cell protoplasts were patch clamped in whole-cell mode with 10 mM K^+ in the bath medium and 150 mM K^+ in the pipette. Current responses to voltage steps from 0 mV holding voltage to test voltages between +120 mV and -100 mV in 20 mV increments were recorded before and after the addition of 100 μg ml^{-1} CHT.

Our results show that CHT affects the time- and voltage-dependent cation currents which are in close relation with stomatal opening and closure (Fig. 3).

Acknowledgements

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